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Summary

Owing to its ability to generate the clot-dissolving protease plasmin, tissue plasminogen activator (tPA) is the only approved drug for the acute treatment of ischemic stroke. However, tPA also promotes hemorrhagic transformation and excitotoxic events. High mobility group box-1 protein (HMGB-1) is a non-histone transcription factor and a pro-inflammatory cytokine, which has also been shown to bind to both tPA and plasminogen. We thus investigated the cellular and molecular effects through which HMGB-1 could influence the vascular and parenchymal effects of tPA during ischemia. We demonstrate that HMGB-1 not only increases clot lysis by tPA, but also reduces the passage of vascular tPA across the blood–brain barrier, as well as tPA-driven leakage of the blood–brain barrier. In addition, HMGB-1 prevents the pro-neurotoxic effect of tPA, by blocking its interaction with N-methyl-D-aspartate (NMDA) receptors and the attendant potentiation of NMDA-induced neuronal Ca^{2+} influx. In conclusion, we show in vitro that HMGB-1 can promote the beneficial effects of tPA while counteracting its deleterious properties. We suggest that derivatives of HMGB-1, devoid of pro-inflammatory properties, could be used as adjunctive therapies to improve the overall benefit of tPA-mediated thrombolysis following stroke.

Key words: Tissue plasminogen activator, High mobility group box-1 protein, Excitotoxicity, Stroke

Introduction

The serine protease tissue plasminoNR1gen activator (tPA) is one of two mammalian proteases that convert plasminogen into active plasmin, leading to fibrin degradation (Vassalli et al., 1991). This has been the rationale for the development of a recombinant form of tPA (Actilyse®), which remains to date the only approved drug for the acute treatment of ischemic stroke (1995). Unfortunately, the achievable benefit of tPA-induced thrombolysis is limited by a narrow therapeutic window (Hacke et al., 2008) and a risk of hemorrhagic transformation (The National Institute of Neurological Disorders and Stroke rt-PA Stroke Study Group, 1995; Saqqur et al., 2008). Concordant experimental data also suggests that tPA (endogenous and exogenous) might endanger endothelial and neuronal cells, leading to an alteration of the integrity of components of the neurovascular unit (for a review, see Yepes et al., 2009). Moreover, intravenously injected tPA can cross the intact or damaged blood–brain barrier (BBB) and thus promote excitotoxic brain damage in rodents (Benchenane et al., 2005a; Lopez-Atalaya et al., 2007).

Since the pioneering work of Tsirka and colleagues (Tsirka et al., 1995), which demonstrated that endogenous tPA has a role in aggravation upon excitotoxic neuronal death, several plasminogen-dependent and -independent mechanisms have been proposed to explain the deleterious effects of this protease in the ischemic brain (for a review, see Samson and Medcalf, 2006; Wang et al., 2008; Yepes et al., 2009). We (Benchenane et al., 2007; Fernandez-Monreal et al., 2004; Nicole et al., 2001) and others (Kvajo et al., 2004; Samson et al., 2008) have proposed that tPA is able to cleave the GluN1 subunit of the glutamatergic N-methyl-D-aspartate (NMDA) receptors, either directly (Nicole et al., 2001) and/or through cooperation with a third partner (Samson et al., 2008). This proteolysis occurs on the extracellular amino-terminal domain (ATD) of GluN1 and leads to an increase in Ca^{2+} influx through activated NMDA receptors to potentially toxic levels (Fernandez-Monreal et al., 2004; Nicole et al., 2001). More recently, we have proposed a 3D model of this interaction of tPA with the NMDA receptor GluN1 subunit, which occurs by a two-site 'docking system', in which the binding of the lysine binding site (LBS) of the Kringle-2 domain of tPA to one unit of a GluN1 dimer is the crucial step for subsequent proteolysis (Lopez-Atalaya et al., 2008). Together, these observations highlight the multifaceted aspects of tPA in an ischemic context, with both beneficial and deleterious effects, depending on the compartment, the timing and the target protein.

High mobility group box-1 protein (HMGB-1), which is also called amphoterin, is a non-histone DNA binding protein composed

of a C-terminal tail and two HMG boxes (A and B) that are DNA binding domains (Landsman and Bustin, 1993). First described as a chromosomal protein, HMGB-1 has been implicated in diverse intracellular functions including the stabilization of nucleosomal structure and the facilitation of gene transcription (Bustin, 1999). In addition, HMGB-1 was described as a cytokine-like mediator of delayed endotoxin lethality and inflammation (Abraham et al., 2000; Bonaldi et al., 2003; Wang et al., 1999). In the brain, HMGB-1 is synthesized by neurons and astrocytes and has been reported to be released after cytokine stimulation and to elicit inflammatory processes (Agnello et al., 2002; Wang et al., 1999). However, because HMGB-1, through its LBS, has been reported to bind both tPA and plasminogen, resulting in an increased transformation of plasminogen into active plasmin (Parkkinen and Rauvala, 1991), we postulated that HMGB-1 could be an interesting modulator of the effects of tPA during ischemic stroke.

In this study, we demonstrate in vitro that by interacting with tPA, HMGB-1 can reverse the side effects of tPA, including its ability to cross the BBB and to promote BBB leakage and neuronal death, while concurrently promoting the beneficial fibrinolytic activity of tPA. Thus, although HMGB-1 is known to promote inflammatory processes following stroke (Hayakawa et al., 2008; Kim et al., 2006; Liu et al., 2007; Qiu et al., 2008; Qiu et al., 2010), our work provides evidence that HMGB-1 can counteract the deleterious effects of tPA and therefore should be considered as a new therapeutic strategy for stroke.

Results

HMGB-1 increases tPA-mediated plasmin formation and blood clot lysis

We first investigated the influence of recombinant HMGB-1 on the ability of recombinant tPA to convert plasminogen into active plasmin. When compared with tPA alone (0.5 μM), tPA and recombinant HMGB-1 together led to a dose-dependent increase in plasmin formation (+30%, +62% and +130% at 1, 2 and 4 μM HMGB-1 respectively; $P<0.01$; $n=6$) (Fig. 1A). Addition of HMGB-1 alone did not promote plasmin formation (data not shown). Accordingly, in an in vitro human plasma clot lysis assay, tPA alone induced clot lysis in a dose-dependent manner, an effect promoted by the addition of recombinant HMGB-1 (Fig. 1B). Interestingly, addition of equimolar HMGB-1 to the lowest dose of tPA tested (15 nM) resulted in a similar efficiency of clot lysis as the highest dose of tPA alone (75 nM; $n=3$, $P<0.05$) (Fig. 1B). Thus, as previously suggested (Parkkinen and Rauvala, 1991), we show that HMGB-1 can promote tPA-dependent fibrinolysis.

HMGB-1 decreases the passage of tPA through the BBB

In accordance with previous observations (Benchenane et al., 2005a; Benchenane et al., 2005b), we confirm by using an in vitro model of the BBB (Fig. 2A), that tPA can cross the BBB under normal conditions and that this passage is increased under ischemic-like conditions (oxygen and glucose deprivation, OGD). Under control conditions, HMGB-1 (0.3 μM) significantly reduced the passage of tPA (–30%, $P<0.05$, $n=3$) from the luminal ('vascular') to the abluminal ('parenchymal') compartment, as demonstrated by zymography and its corresponding densitometric analyses (Fig. 2B). None of these conditions altered the permeability of the BBB (Fig. 2D, control conditions). Under OGD conditions, the passage of tPA alone was dramatically increased (+482% compared with tPA alone in control conditions $P<0.05$, $n=3$), yet in the presence of HMGB-1, this passage was significantly decreased (–20% versus the passage of tPA alone under OGD conditions, $P<0.05$, $n=3$) (Fig. 2C). tPA aggravated OGD-induced BBB permeabilization (passage of Lucifer Yellow increased by 132% $P<0.001$, $n=6$; Fig. 2D), but this alteration of BBB integrity was partially prevented by the co-administration of HMGB-1 (–58% versus tPA alone, $P<0.001$, $n=6$; Fig. 2D).

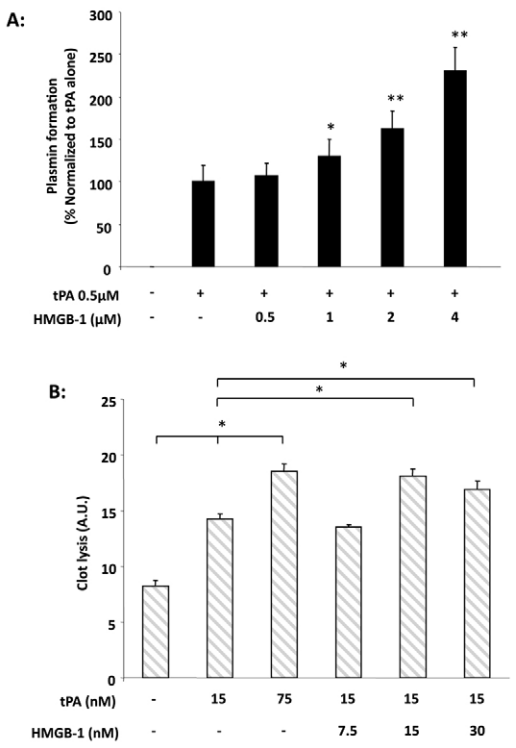

Fig. 1. Effects of tPA and HMGB-1 on plasminogen to plasmin conversion and clot lysis. (**A**) Plasmin formation was measured with a specific chromogenic substrate in the presence of tPA (0.5 μM) and HMGB-1 at different concentrations (0 to 4 μM) (n= six per group). *$P<0.05$, **$P<0.01$ compared with tPA 0.5 μM. (**B**) Effect of tPA (15 or 75 nM) alone or together with HMGB-1 (7.5, 15 and 30 nM) on human clot lysis performed by measuring fluorescence released from a pre-formed clot as described in the methods section (n= six per group). *$P<0.05$.

HMGB-1 reverses the pro-neurotoxic effect of tPA

To determine whether HMGB-1 could influence the ability of tPA to promote NMDA-induced neuronal death, we used a model of excitotoxicity induced by exposure of primary cortical neuronal cultures to NMDA (10 μM, 24 hours). Whereas NMDA produced acute cell body swelling, followed 24 hours later by neuronal degeneration (30%), as measured by the release of lactate dehydrogenase in the bathing medium, neither tPA (0.3 μM) nor HMGB-1 (0.3 μM) alone induced neuronal death. However, although tPA caused a twofold increase in NMDA-induced neuronal death ($n=10$, *$P<0.05$), co-application of HMGB-1 prevented the pro-excitotoxic effects of tPA on NMDA-induced neuronal death (Fig. 3A).

It was previously demonstrated that tPA increases excitotoxicity by interacting with, and cleaving the ATD-GluN1 subunit of NMDA receptors (Benchenane et al., 2007; Nicole et al., 2001). Here, we performed immunoblots after incubation of the recombinant form of ATD-GluN1, with tPA and/or HMGB1. We observed that after

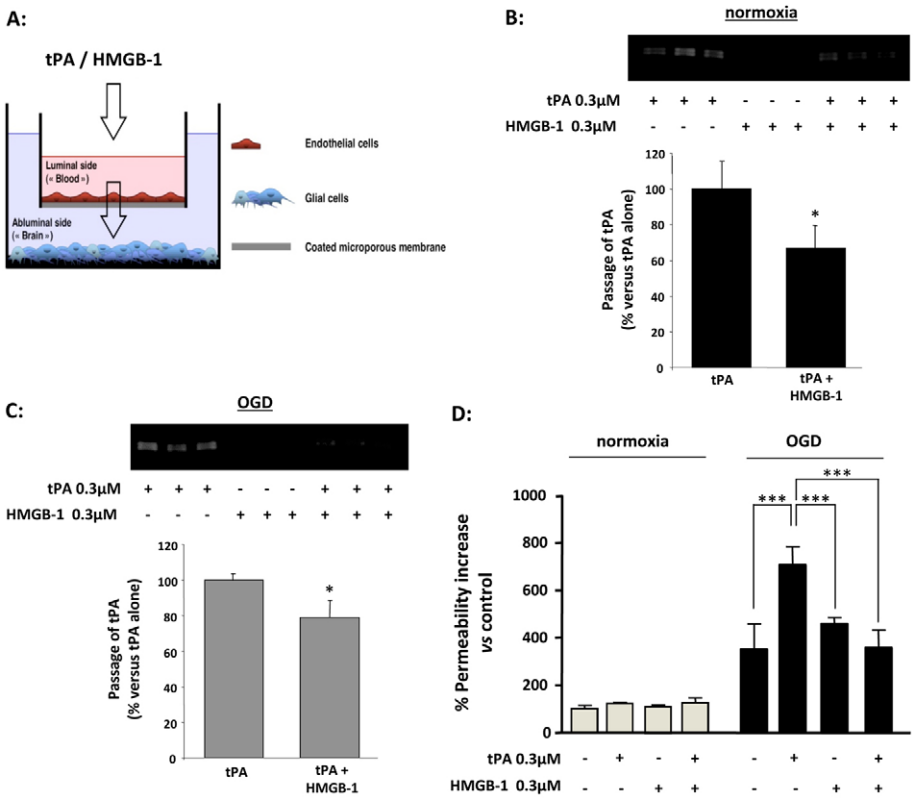

Fig. 2. HMGB-1 reduces the passage of tPA across the BBB in vitro in both control and OGD conditions. (**A**) tPA alone (0.3 µM) or in the presence of HMGB-1 (0.3 µM) was applied on the upper endothelial compartment. (**B**,**C**) tPA activity was measured after 2 hours in the lower medium by zymography assay both in control and OGD conditions. Densitometries are normalized to rtPA (mean values ± s.e.m. of three experiments per group; **P*<0.05 Mann–Whitney test). In both control and OGD, HMGB-1 decreases passage of tPA through the BBB. (**D**) BBB permeability to Lucifer Yellow (Pe) was not modified by tPA and/or HMGB-1 in control conditions. OGD by itself increases Pe compared with normoxia and is potentiated by tPA. HMGB-1 does not change Pe under OGD conditions but prevents tPA increasing Pe. Results are presented as mean ± s.e.m. The 100% control value represents a Pe of 0.40×10^{-3} cm/minute for LY after 4 hours under control conditions without treatment. The mean scores were significant using one-way ANOVA (****P*<0.001; *n*= six monolayers per treatment, Bonferroni's test).

tPA treatment, recombinant ATD-GluN1 was cleaved, whereas this peptide remained unaffected after the co-application of tPA+HMGB-1 (Fig. 3B) (*n*=3). Using pull-down assays performed with antibodies directed against either HMGB-1 or GluN1, we observed no evidence that HMGB-1 complexes with GluN1 (Fig. 3C), thus ruling out the possibility that the prevention of ATD-GluN1 cleavage by tPA in the presence of HMGB-1 was due to a competition for the binding to ATD-GluN1.

HMGB-1 prevents tPA from potentiating NMDA-evoked Ca^{2+} influx

We then investigated whether the effects observed in the excitotoxic neuronal death paradigm were correlated with a modulation of NMDA-evoked Ca^{2+} influx, which is one of the crucial events in excitotoxic necrosis. By using Fura-2 fluorescence videomicroscopy (Fig. 4A,B), we found that tPA (0.3 µM for 15 minutes) increased NMDA-induced Ca^{2+} influx in cultured cortical neurons by approximately 45%, whereas the co-application of tPA and HMGB-1 (0.3 µM each for 15 minutes) prevented the potentiation of Ca^{2+} influx in neurons (*n*=5 independent experiments for a total of 140 cells per condition). This was not due to an effect of HMGB-1 by itself, because it did not alter the extent of NMDA-evoked Ca^{2+} responses (data not shown). These results are in agreement with the observation that HMGB-1 prevents both the cleavage of ATD-GluN1 and the pro-excitotoxic effects induced by tPA.

Discussion

In addition to its nuclear functions (for a review, see Bustin, 1999), HMGB-1 is reported to act as a pro-inflammatory cytokine or mediator (Rouhiainen et al., 2007; Wang et al., 1999) and was thus considered as a deleterious molecule in experimental models of cerebral ischemia (Kim et al., 2006; Liu et al., 2007; Muhammad et al., 2008). However, stroke pathogenesis is complex, involving not only local and peripheral inflammatory events, but also a set of cascades that challenge the integrity and survival of all components of the neurovascular unit (for a review, see Dirnagl et al., 1999). The first emergency treatment of ischemic stroke is to restore cerebral blood flow, with tPA-induced reperfusion being the only approved treatment (The National Institute of Neurological Disorders and Stroke rt-PA Stroke Study Group, 1995). Nevertheless, it is now well known that tPA is a multifaceted molecule, displaying both beneficial (thrombolytic activity in the vascular compartment) and deleterious effects (promotion of BBB leakage, hemorrhagic transformation, and neuronal and endothelial cell death) (for a review, see Yepes et al., 2009). The positive and negative effects concern both endogenously produced and intravenously administered tPA, which has also been shown to cross the BBB, even when BBB integrity is not compromised (Benchenane et al., 2005a; Benchenane et al., 2005b). Here, in a set of well-characterized in vitro models, we show that in the presence of HMGB-1, the fibrinolytic activity of tPA is significantly increased, whereas its ability to cross the BBB and to promote BBB leakage and excitotoxicity are significantly reduced.

The rationale for the use of tPA for acute ischemic stroke treatment, relies on its ability to convert plasminogen into active plasmin, the trypsin-like serine protease driving the enzymatic dissolution of fibrin clots. As a result of its high affinity for fibrin, tPA activates clot-bound plasminogen 100-times more efficiently than it does circulating plasminogen (Tanswell et al., 1989). However, considering the high dose of tPA administered for thrombolysis in stroke patients (0.9 mg per kg of body weight; 10% given as a bolus followed by constant infusion of the remaining 90% over 1 hour), one can estimate that a significant proportion of the protease can exert effects other than the expected

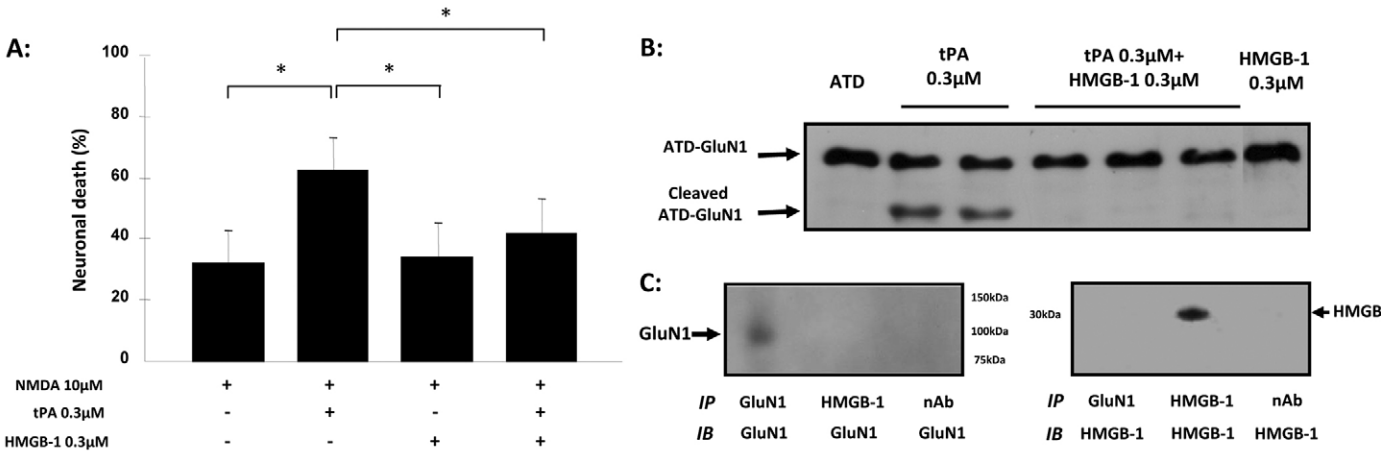

Fig. 3. Effect of tPA–HMGB-1 complexes on neurotoxicity mediated by tPA. (**A**) Neuronal death was quantified by measuring LDH release into the medium after a 24 hour exposure to NMDA (10 µM) in the presence or absence of tPA (0.3 µM) and HMGB-1 (0.3 µM). The co-application of HMGB-1 counteracts the pro-excitotoxic effects of tPA (n=16, *P<0.05). (**B**) HMGB-1 prevents tPA from cleaving the recombinant form of the N-terminal domain of the NMDA receptor subunit (ATD-GluN1) (three independent assays). (**C**) The prevention of tPA-mediated cleavage of the ATD-GluN1 is not due to an interaction between tPA and NMDA receptor as shown by immunoprecipitation experiments using antibodies against GluN1 or HMGB-1. IP, immunoprecipitation; IB, immunoblot.

thrombolysis, such as bleeding. Accordingly, it might be crucial to reduce the doses of tPA to limit these side effects and in this view, our finding that HMGB-1 potentiates the ability of tPA to convert plasminogen into plasmin, and thus promotes clot lysis, opens an interesting area of investigation.

We have previously demonstrated both in vitro and in vivo, that exogenous tPA crosses the intact BBB through a low-density-lipoprotein-related receptor protein (LRP)-dependent transcytosis and thus promotes excitotoxic neuronal death (Benchenane et al., 2005a; Benchenane et al., 2005b). In addition, following OGD, a condition known to mimic in vitro the ischemic condition at the stage of occlusion, which induces an early increase of BBB permeability (Brillault et al., 2002; Mysiorek et al., 2009), we demonstrated that the transport of tPA across the BBB was dramatically increased and that the mechanism of passage shifted from an LRP-dependent process to an LRP-independent one (Benchenane et al., 2005b). The interaction of tPA with LRP at the parenchymal level of the neurovascular unit has also been associated with edema formation and activation of metalloproteases, including MMP-9, in addition to subsequent hemorrhagic transformation (for a review, see Yepes et al., 2009). Here, we observed that HMGB-1 significantly decreases the passage of tPA across our model of BBB (under normal or ischemic-like conditions). Because HMGB-1 alone did not reduce OGD-induced BBB leakage, our study suggests that by physically interacting with tPA, HMGB-1 prevents LRP-dependent and -independent passages of tPA, thus indirectly protecting the BBB. This suggests that an adjunct therapy coupling HMGB-1 to tPA might reduce the extent of BBB leakage and edema in stroke patients.

Previously, HMGB-1 has been shown to increase neuronal death induced by exposing mixed cultures of neurons and glial cells to low doses of glutamate, whereas HMGB-1 had no effect in high doses of glutamate (Faraco et al., 2007). The glutamate dose-dependent effect of HMGB-1 was not reversed by addition of an anti-inflammatory agent, suggesting a role for HMGB-1 in glutamate-mediated neurotoxicity. In our model of primary cultures of pure cortical neurons, HMGB-1 did not influence NMDA-mediated neurotoxicity (Fig. 3A), but specifically protected neurons from the pro-excitotoxic effects of tPA (Benchenane et al., 2007; Nicole et al., 2001). This neuroprotection results from the prevention of the physical interaction between tPA and the NMDA receptor subunit GluN1, an interaction that otherwise results in increased Ca^{2+} influx through activated NMDA receptors (Nicole et al., 2001; Samson et al., 2008). Our recent demonstration that the deleterious effect of tPA on NMDA-receptor-mediated neuronal loss is dependent on its kringle-2 domain (Lopez-Atalaya et al., 2008), together with the demonstration that HMGB-1 interacts with the lysine binding sites within the kringle-2 domain of tPA (Parkkinen and Rauvala, 1991), suggest that HMGB-1 protects neurons by occupying the site of interaction of tPA with the GluN1 subunit.

Altogether, our in vitro data suggest that HMGB-1 can potentially exert several beneficial effects during stroke: a potentiation of tPA-dependent fibrinolysis and the blockage of tPA-dependent parenchymal side effects. However, HMGB-1 has been reported to promote expression of pro-inflammatory cytokines such as TNF (Rouhiainen et al., 2007), and inflammation is currently considered to be a deleterious pathway in a stroke context. Accordingly, it was demonstrated, in vivo, that HMGB-1 could promote delayed inflammatory processes in the post-ischemic brain, with global deleterious effects (Kim et al., 2006). Surprisingly, in this work, the authors performed injections of short hairpin RNA against HMGB-1 into the striatum, despite reporting decreased levels of HMGB-1 due to ischemia. Yet, their conclusions were that HMGB-1 aggravates ischemic damage to the brain by promoting late inflammatory processes. In another study, Liu and colleagues demonstrated in rats that a monoclonal antibody against the acidic tail of HMGB-1 led to a 90% reduction in infarct size after the occlusion of the middle cerebral artery, which resulted in an overall improvement of the neurological outcome (Liu et al., 2007). The antibody was injected intravenously immediately or 6 hours after the initiation of mechanical reperfusion. The authors correlated the reduction in infarct size with a decrease in inflammatory markers and activation of metalloproteinases such as MMP-9 (Liu et al., 2007). Similar conclusions were reported when this antibody was used in a model

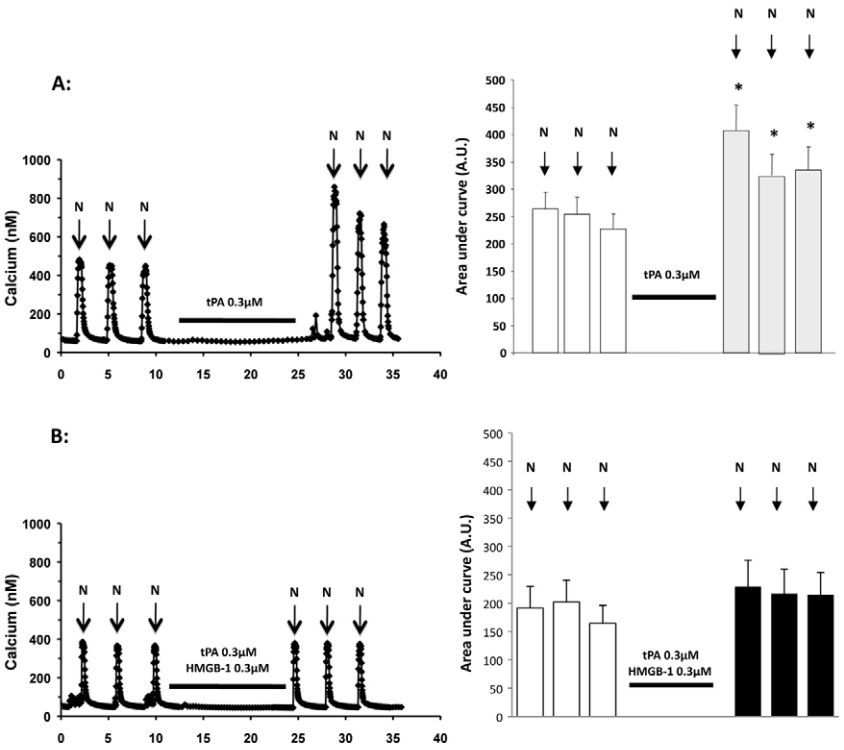

Fig. 4. HMGB-1 prevents tPA-promoted NMDA-induced neuronal Ca^{2+} influx. (**A**) Three successive 30 second exposures (N) to NMDA (10 μM) led to a rapid and reproducible Ca^{2+} influx in cultured cortical neurons (*n*=3). Cells were then incubated for 15 minutes with tPA alone or with HMGB-1 and NMDA exposure performed again. (**B**) In contrast to tPA alone (0.3 μM), when co-incubated with HMGB-1 (0.3 μM), tPA does not promote NMDA-induced Ca^{2+} influx in neurons. **P*<0.05 compared with control exposure with NMDA only; tested by ANOVA for repeated measures followed by a Bonferroni–Dunn's test for multiple comparisons.

of focal permanent ischemia (Muhammad et al., 2008). Of note, none of these studies investigated tPA-mediated thrombolysis, a major parameter with regard to the clinical setting (Kim et al., 2006; Liu et al., 2007; Muhammad et al., 2008). In our study, we show the role of HMGB-1 as a catalyst for tPA-driven thrombolysis.

Despite the apparent contradiction with these in vivo studies (mainly suggesting delayed pro-inflammatory effects in the vasculature), our in vitro findings suggest that HMGB-1 can exert beneficial effects during ischemic stroke (by preventing BBB leakage, increasing clot dissolution and reducing excitotoxic neuronal loss). Interestingly, it was previously demonstrated that HMGB-1 binds to tPA through its Box-A domain (Parkkinen and Rauvala, 1991), suggesting that the C-tail, which is known to mediate the inflammatory effects of HMGB-1 (Liu et al., 2007; Muhammad et al., 2008; Rouhiainen et al., 2007; Sha et al., 2008; Tian et al., 2007), might not be necessary for the beneficial effects of HMGB-1. However, the recent demonstration that inflammatory cytokines, such as IL-1β, can contaminate the purified product during some HMGB-1 purification processes, adds a note of caution to the interpretation of some data (Sha et al., 2008). In a recent review, it has been suggested that HMGB-1 could have biphasic actions during stroke: an early and noxious inflammatory effect, but delayed beneficial actions, including stem cell migration into the ischemic region, and promotion of neurogenesis, vasculogenesis and angiogenesis (Hayakawa et al., 2010a; Hayakawa et al., 2010b). Here, our results implement this work, by demonstrating the pro-thrombolytic and protective effect of HMGB-1 for the BBB and neurons.

Overall, the literature argues against the direct use of HMGB-1 because of pro-inflammatory properties. However, we provide evidence here suggesting that injection of HMGB-1 into the circulation might concurrently promote tPA-dependent fibrinolytic activity and prevent its passage across the BBB. Moreover, if vascular HMGB-1 can cross the BBB, its injection could help to prevent the pro-neurotoxic effects of tPA. However, a clear demonstration of the passage of HMGB-1 remains to be established because conflicting results have been reported (Kim et al., 2006; Muhammad et al., 2008; Qiu et al., 2008). In addition, the impermeability to HMGB-1 of the insert used in our in vitro model of BBB did not allow us to investigate this question (data not shown). Despite this, endogenous HMGB-1 released in the brain parenchyma might at least prevent the pro-neurotoxic effects of tPA, as well as its damaging effect on the BBB. Development of HMGB-1-derived molecules devoid of inflammatory actions could be an alternative safer strategy. In line with this hypothesis, it has been shown in a stroke model that Box-A of HMGB-1 prevents the binding of HMGB-1 to RAGE (receptor for advanced glycation end products), leading to a decrease in infarct volume (Muhammad et al., 2008). Understanding which part(s) of the HMGB-1 molecule is involved in each of these processes is thus mandatory to the development of a more effective therapy for the treatment of stroke.

Materials and Methods

Reagents

tPA was purchased from Boehringer Ingelheim. Recombinant HMGB-1 was produced and purified as described previously (Parkkinen and Rauvala, 1991). Briefly, HMGB-1 was produced in a baculovirus system on a 5 ml heparin-Sepharose Hi-Trap-column (Pharmacia). The column was eluted with a linear gradient of 0.15–1.5 M NaCl in 50 mM Tris-HC1, pH 8.5. Fractions containing recombinant HMGB-1 were pooled and subjected to a second round of purification on heparin-Sepharose under the same conditions.

Cell culture

Primary cultures of cortical neurons were prepared from fetal mice (E15-E16). Dissociated cortical cells were resuspended in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum, 5% horse serum, and 1 mM glutamine, and plated in 24-well dishes previously coated with poly-D-lysine and laminin. After 3 days in vitro (DIV), the cells were exposed to 10 μM Ara-C to inhibit glial proliferation. Cultures were used after 14 DIV for excitotoxicity and Ca^{2+} assays.

Excitotoxicity

Slowly triggered excitotoxicity was induced at 37°C by a 24 hour exposure to 10 μM NMDA in DMEM supplemented with glycine (10 μM). Neuronal death was estimated by phase-contrast microscopy and quantified by measurement of lactate dehydrogenase (LDH) release by damaged cells into the bathing medium. The LDH level corresponding to complete neuronal death was determined in sister cultures exposed to 200 μM NMDA for 24 hours in DMEM supplemented with glycine. Background LDH levels were determined in sister cultures subjected to sham wash and subtracted from experimental values to yield the signal specific to experimentally induced injury.

Measurement of tPA-dependent conversion of plasminogen into plasmin

tPA (2 μg/ml) was incubated for 1 hour at 22°C in fibrin-coated microtitre plates followed by three PBS washes to remove non-fixed tPA. Plasminogen (0.3 μM) alone, or in combination with increasing concentrations of HMGB1 (0.5 to 4 μM), were added. The kinetics of plasmin formation was followed for 2 hours by measuring the release of para-nitroaniline from the plasmin sensitive chromogenic substrate CBS0065 (0.75 mM; Diagnostica Stago, France) by measuring its absorbance at 405 nm.

In vitro human clot lysis

A pool of human plasma was given by l'Etablissement Français du Sang. 500 μl of human plasma, 20 mM $CaCl_2$, and 0.1 mg/ml fibrinogen labeled with Alexa Fluor 647 (Molecular Probes) were combined. Human thrombin (Sigma, France) at 1 NIHU/ml was added to promote coagulation. Plasma solutions were incubated for 2 hour at 37°C to promote clot formation. For clot lysis, 2 ml of human plasma containing tPA and/or HMGB-1 at varying concentrations were added, and fluorescence activity measured every hour by measuring free Alexa Fluor 647 (InVitrogen, France) released into the supernatant.

Immunoblotting

Lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 0.5% Triton X-100) containing protease and phosphatase inhibitor cocktails (1:100, Sigma) and PMSF (1 mM), was used to solubilize cell lysates. Supernatants were harvested after centrifugation at 9000 ***g*** for 10 minutes and protein concentrations were estimated with the BCA assay (Pierce, Rockford, IL). Protein samples were separated by SDS-PAGE and transferred onto a PVDF membrane. Membranes were blocked for 2 hours in 5% dried milk and Tris-buffered saline containing 0.1% Tween-20 and incubated overnight at 4°C with the primary antibody (Amphoterin, 1:3000; Sigma). Biotinylated secondary antibody (1:10,000) was added for 1 hour followed by incubation at room temperature with peroxidase-conjugated streptavidin (1:2500) for 1 hour. Proteins were detected with a chemiluminescence ECL Plus immunoblotting detection system (Perkin Elmer-NEN, Paris, France).

Ca^{2+} videomicroscopy analysis

Primary cultures (14 DIV) of cortical neurons were loaded in the presence of a HEPES-buffered saline solution containing 5 μM Fura-2/AM and 0.1% pluronic F-127 (Molecular Probes, Leiden, The Netherlands) (45 minutes, 37°C). Experiments were performed at 22°C on a Nikon Eclipse inverted microscope equipped with a 75W Xenon lamp and a Nikon ×40, 1.3 numerical aperture epifluorescence oil-immersion objective. Fura-2 (excitation: 340, 380 nm, emission: 510 nm) ratio images were acquired with a CCD camera (Princeton Instrument, Trenton, NJ), and digitized (256×512) using Metafluor 4.11 software (Universal Imaging Corporation, Cherter, PA).

Construction of His-tagged recombinant ATD-GluN1 domain

The region of the GluN1 subunit encoding amino acids 19–371 of GluN1-1a corresponding to the ATD was amplified from the full-length rat GluN1-1a cDNA by the upstream primer 5′-cgggatcccgcgccgcctgcgac-3′ (generating a *Bam*HI restriction site) and the downstream primer 5′-atgggtaccattgtagatgcccac-3′ (containing an internal *Kpn*I restriction site). PCR products were digested and inserted into the pQE100-Double Tag vector (Qiagen, Courtaboeuf, France) which encodes 6×His at the N-terminus of the insert. Recombinant proteins were purified from inclusion bodies of IPTG-induced bacterial cultures (*E.coli*, M15 strain) on a nickel affinity matrix as described by the manufacturer (Qiagen, Courtaboeuf, France) and analysed either by silver or Coomassie Blue staining after SDS-PAGE.

Immunoprecipitation

Recombinant proteins were incubated at 4°C for 1 hour before immunoprecipitation. Proteins were incubated with ATD antibody for 2 hours at 4°C (1/1000 Penta-His, Qiagen, France) followed by adsorption to protein G-Sepharose (Amersham, France). Bound proteins were eluted in DTT and β-mercaptoethanol sample buffer and separated with SDS-PAGE electrophoresis.

Zymography assays

Zymography was performed by adding plasminogen (4.5 μg/ml) and casein (5 mg) to a 12.6% SDS-PAGE. Electrophoresis was performed at 4°C and gels were washed with Triton X-100 (2.5%) and incubated at 37°C. Caseinolytic bands were visualized with Coomassie Blue staining.

In vitro model of BBB

The method of Dehouck and colleagues (Dehouck et al., 1990) was used with minor modifications. Bovine brain endothelial cells isolated from capillary fragments were co-cultured with primary mixed glial cells from newborn Sprague–Dawley rats. The glial cells were isolated and cultured for 3 weeks, plated on the bottom of cell culture clusters containing six wells each. The endothelial cells were seeded onto cell culture inserts, which were placed in the wells containing glial cells. The medium used for the co-culture was DMEM supplemented with 10% calf serum, 10% horse serum, 2 mM glutamine, 50 mg/l gentamicin, and 1 μg/l basic fibroblast growth factor (Sigma). The medium was changed every 2 days and under these conditions, endothelial cells formed a confluent monolayer after 7 days. Experiments were performed 5 days after confluency.

Transport studies in control conditions

Permeability studies were performed using Lucifer Yellow (LY) (Lucifer Yellow CH dilithium, Sigma), recombinant HMGB-1, or recombinant tPA. LY is a non-radioactive surrogate marker of BBB integrity, with a good correlation with other markers such as sucrose permeability and integrity of tight junction proteins (Culot et al., 2009; Mysiorek et al., 2009). In these studies, endothelial cell monolayers were transferred to six-well plates containing 2.5 ml Ringer–HEPES solution (150 mM NaCl, 5.2 mM KCl, 2.2 mM $CaCl_2$, 0.2 mM $MgCl_2(6H_2O)$, 6 mM $NaHCO_3$, 5 mM HEPES, 2.8 mM glucose, pH 7.4) per well. 1.5 ml Ringer–HEPES solution containing LY at 50 μM and 20 mg/l tPA (only for tPA transport). Incubations were performed at 37°C. At 20, 40 and 60 minutes (for LY), or after 2 hours for tPA and HMGB-1, an aliquot from each lower compartment and stock solution was taken. Quantification of fluorescence was performed with an aliquot of 200 μl withdrawn from each abluminal compartment at each time and 20 μl of luminal compartment and initial solution (Fluoroskan Ascent FL Thermolabsystems). tPA activity in the abluminal compartment was determined by zymography as previously described. For LY, triplicate control wells were also assayed to determine the endothelial permeability coefficient (Pe) calculated in centimeters per minute as previously described (Dehouck et al., 1992). In this calculation, both filter permeability (Psf=insert filter + collagen coating) and filter plus cell permeability (Pst=filter + collagen + endothelial cells) were taken into account, according to the formula: 1/Pe=1/Pst–1/Psf.

For each experimental condition, data were mean values ± s.e.m. (n=6 experiments) of LY Pe (in cm/minute) passage through the endothelial monolayer. All transport data were expressed as a percentage of the control.

Transport studies during OGD

OGD transport studies were performed as described above. DMEM and Ringer–HEPES solutions were first equilibrated with nitrogen and glucose was removed from the medium (Brillault et al., 2002). Cells were incubated in Gas Pack EZ bags (Becton Dickinson, Sparks, MD) for the indicated time and transport studies were performed in an airtight glove box filled with nitrogen at 37°C (Forma Scientific, Mountain View, CA).

Statistical analysis

Results are expressed as the mean ± s.e.m. Statistical analyses were performed by a Kruskal–Wallis test followed by a Mann–Whitney test, and Bonferroni test for LY permeability studies.

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